

EFFECTS OF *Glomus mosseae* AND *Pseudomonas fluorescens* ON ECO-PHYSIOLOGICAL TRAITS AND ANTIOXIDANT PRODUCTION OF MUNG BEAN UNDER DROUGHT CONDITION

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ABSTRACT

Drought is one of the most critical environmental stressors affecting agricultural productivity around the world and it considerably results in yield. Symbiosis interaction between plants and soil micro-organisms are considered to enhance plant tolerance in a/biotic conditions. In this study, the impact of *Glomus mosseae*, *Pseudomonas fluorescens* strain 169 and both of them under imposed water stress (flowering and pod filling stages) of mung bean (*Vigna radiata* (L.) Wilczek) was evaluated in two farm experiments during 2016 and 2017. Eco-physiological parameters have been recorded which showed that drought stress reduced the number of leaves, root colonization and seeds yield of mung bean. Mixed inoculation of *G.mosseae* and *P.fluorescens* 169 was more effective in alleviation the harm effects of drought stress. Enzymes assay suggested that co-inoculation of *G.mosseae* and *P.fluorescens* 169 was more effective to increase antioxidative defense system like catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) activities. H₂O₂ contents were increased by water stress both in cutting irrigation at flowering and pod filling stages. In conclusion, plants inoculated with combination of *G.mosseae* and *P.fluorescens* 169 had less oxidative damage over control plants.

Key words: *Glomus mosseae*, Mung bean, Oxidative damage, *Pseudomonas fluorescens*, Root colonization, Symbiosis, *Vigna radiata*, Water deficit

INTRODUCTION

One of the major abiotic threats on agriculture productivity is the progressive diffusion of water deficit in different areas of the world (Dai, 2011), which induces a reduction in plant growth and development (Toker and Mutlu, 2011). Drought has a negative impact on nutrient supply, leading to a decrease in phosphate availability (Sardans and Peñuelas, 2004). Although drought impedes on wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) performance in all growth stages, it is more critical during the flowering and grain-filling phases (terminal drought) and results in substantial yield losses. (Toker et al., 2009; Farooq et al., 2014). Ganbari and Mollashahi Javan (2015) suggested that irrigation in pod filling stage improved final seed yield of mung bean (*Vigna radiata* (L.) Wilczek). Among the studies on drought, great interest has been developed in the last years to beneficial soil micro-organisms, including arbuscular mycorrhizal fungi (AMF) with a role to improve plant nutrition and tolerance to several environmental stresses. AM symbiosis is one of the most ancient interactions between the roots of more than 80% of terrestrial plants, including several crops

(Balestrini and Lumini, 2018). AM fungi can promote plants performance and protect them from various stresses, including water deficit (Lenoir et al., 2016; Balestrini and lumini, 2018). Some of the bacterial strains mitigating biotic stresses have also been shown to protect plants against abiotic stresses and promote plant growth by improving soil structure and moisture retention as well as by enhancing plant mineral-nutrition absorption (Bashan et al., 2013; Kim et al., 2012). Treatment with *Pseudomonas fluorescens* enhanced the growth parameters of *Catharanthus roseus* under drought stress and partially ameliorated the drought induced growth inhibition by increasing the fresh and dry weights significantly (Abdul Jaleel et al., 2007). Microbial communities below the ground influence the selection on plant traits by mitigating the effects of abiotic stress on plant population (Lau and Lennon, 2011). Manipulation of below-ground microbial communities showed that *Brassica rapa* grown in soils with simplified microbial communities were smaller, with reduced chlorophyll content, fewer flowers, and less fecund when compared with plants population grown in association with more complex soil microbial communities

(Lau and Lennon, 2011). AMF and bacteria can interact synergistically to stimulate plant growth through a range of mechanisms that include improved nutrient acquisition (Barea et al., 2002) and enhancement of root branching (Gamalero et al., 2004). Only a few strains of bacteria with mycorrhizal fungi can be used effectively and show synergistic effects (Ruiz-Lozano and Bonfante, 2001). The performance of flaxseed (*Linum usitatissimum* L.) partially enhanced by microbial dual inoculations as well as mycorrhizal and/or phosphate solubilizing bacteria (Rahimzadeh and Pirzad, 2019). Bianciotto et al. (1996) reported that some *Rhizobium* and *Pseudomonas* species attached to germinated AM fungal spores and hyphae under sterile conditions, and that the degree of attachment varied from the bacterial strains. Therefore, some microorganisms, named mycorrhiza helper bacteria (MHB) can facilitate establishment and the functioning of AMF symbiosis by stimulating spore germination, mycelial growth, root colonization or sporulation, but also by reducing stresses that could impact on AMF symbiosis (Leis et al., 2018).

Activated oxygen compound produced in different compartments of the plant cell such as the chloroplast, mitochondria and peroxisomes for example H_2O_2 , O_2^- , and OH^- may accumulate during water deficit stress and damage the photosynthetic apparatus. Oxidative molecules initiate damage in the chloroplast and cause a cascade of damaging effect including chlorophyll destruction, lipid peroxidation and protein loss (Zhang and Kirkham, 1994). H_2O_2 and malondialdehyde levels were increased by water stress both in arbuscular mycorrhizal and non-mycorrhizal plants, but this increment was significantly highest in NM-plants (Mohamed et al., 2014). More researches approved that there is strong and significant relationship between different oxidative damages caused by environmental stresses and increment in antioxidant activities in photosynthetic plants (Heidari and Golpaygani, 2011). Antioxidative enzymes like catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) are the most important components in the scavenging system of reactive oxygen species (Notor and Foyer, 1998; Rasool et al., 2013). Catalase (CAT) activity was higher under drought stress in mycorrhized plants than those of non-mycorrhizal ones and mixed AMF inoculation had more beneficial effect on plant growth than single AMF inoculation (Zhang et al., 2019). The differences of this study from previous ones is offering new insights for

understanding the physiological mechanisms involved in tolerance toward water deficit as mediated by *G.mosseae* and *P.fluorescens* 169 and a way that mung bean uses to scavenge ROS by enhancing the antioxidant enzymes. We hypothesized that co-inoculation of fungous and bacterium with distinct properties at specific stages of plant development would enhance plant performance.

MATERIALS AND METHODS

Two factorial experiments based on Randomized Complete Block Design with three replications conducted in research and training field of Islamic Azad University, Miyaneh Branch, Iran (37° , $55'N$; 47° , $11' W$; elevation 1100 m) in 2016 and 2017. Experimental plots were drought stress which included; stopping irrigation in flowering stage (D_1), stopping irrigation in pod setting stage (D_2) and normal irrigation (control). In order to calculate the amount of irrigation we used Washington State College (WSC) flume type 3 method which was about 5760000 L/ha. Inoculations as a second factor were included; non-inoculation (Control), inoculation with *G.mosseae*, *P.fluorescens* strain 169 and combination of *G.mosseae*+*P.fluorescens* strain 169.

Partow cultivar of Mung bean was used in this study, which was provided from Seed and Plant improvement Institute, Karaj, Iran. Suspension solution of *P.fluorescens* strain 169 with 10^8 - 10^9 live and active bacteria per ml ($CFUml^{-1}$) was provided from Water and Soil Research Institute, Karaj, Iran. *G.mosseae* was provided from Turan Biotech Company, Iran. It had approximately 330 fungi spores per gram soil and was produced by culturing in host plant which used in form of soil mixed spores and hyphae. *Partow* cultivar of mung bean was sown in 6 rows, 3×1.5 m², with 50 cm inter-row spacing. All agricultural practices done in farm were based on Miyaneh agriculture department guideline. The results of soil physical and chemical analysis of research area indicated that soil structure was clay and amount of organic carbon, nitrogen, phosphorous and potassium in experimental farm were 1.5%, 0.1%, 5.70 ppm and 301ppm respectively. During the experiment, according to soil physical and chemical analysis, substances as a sufficient amount of fertilizer including super triple phosphate 100 kg/ha and sulphate potassium 200 kg/ha were applied. Monthly total rainfall, average temperatures and atmospheric humidity were given in 2016 and 2017 (Table 1).

Table1. Climate data of the experimental cite in 2016 and 2017.

Parameters	Months				Years
	August	July	June	May	
Average temperature (°C)	28.6	29.2	26.2	21.2	2016
Monthly total rainfall (mm)	3.10	0	0.4	8.3	
Average atmospheric humidity (%)	36	40	34	34	
Average temperature (°C)	27.1	31.4	25.2	17.6	2017
Monthly total rainfall (mm)	5	0	9.4	48.8	
Average atmospheric humidity (%)	49	31	39	41	

Source: Synoptic and Meteorological station of Miyaneh city, Iran

For data collection, the number of leaves were recorded according to sampling of ten plants per plot and seed yield were calculated from central rows of each plot after removing margin lines and then converted to kg per hectare.

To determine phosphorous concentration of seeds after sampling and drying by means of Benton (2001) method then coloring by spectrophotometer and Vanadate-molybdate calculated based on equation (1).

$$P (\%) = (a-b) \times (V / 2000w) \times (100/D.M) \quad (1)$$

a is concentration of phosphorous in sample (mg/lit),

b is concentration of phosphorous in control (mg/lit),

V is final volume of extraction in digestion time (ml),

W is dry seed weight used in digestion (ml) and

D.M is seed dry matter percentage.

Relative water content of leaves: According to Steven et al., (1990) and by means of formula 2, calculated:

$$RWC = (Fw - Dw / Sw - Dw) \times 100 \quad (2)$$

Where Fw is fresh weight,

Dw is drought weight and

Sw is saturated weight.

Chlorophyll content of leaves: from the last leaves of plant and by means of Spad-502 measured and noted.

Root colonization: To determinate root colonization percentage Giovannetti and Mosse (1980) method is used. Based on this method the painted root of plants distributed in a petri dishes which contains square grids then observed under binocular and the number of intersections with vertical and horizontal lines determined. Among these cross-sections, those with colonized root sections counted separately and obtained in a fraction of the total intersections based on Equation (3):

$$\text{Root colonization} = \frac{\text{the number of micorrhizal root intersections with grid}}{\text{the number of all intersections between root and grid}} \times 100 \quad (3)$$

Extraction of enzymes

The leaves of mung bean in each plot were collected and washed with distilled water, and then the surface moisture was cleaned. For enzyme extracts and assays, the fresh leaf samples (0.5 g) were frozen in liquid nitrogen and then grounded into a fine powder with a pre chilled mortar and pestle in 5 mL of ice cold, 50 mM potassium phosphate buffer (pH=7.0), containing 1 mM EDTA, 2 mM ascorbic acid (ASA) and 1% (w/v) soluble polyvinylpyrrolidone (PVPP). After filtration (Millipore, Mitex 0.5 mm), the homogenate was centrifuged at 12000 g for 20 min and the supernatant was collected and used for Catalase activity (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) assays. All operations were done at 4°C (Zhang et al., 2019).

Catalase activity (CAT) was assayed by measuring the rate of disappearance of H₂O₂, according to the method Roland et al., (1952). The reaction buffer (3.0 mL) contained 50 mM buffer (pH=7.0), 200 mM H₂O₂, and 100 µl enzyme extracts. Decrease in H₂O₂ was measured as the decline in optical density at 240 nm for 1 min. One unit of CAT was assayed spectrophotometrically by monitoring the decrease in absorbance of H₂O₂ at 240 nm by 0.1 unit per minute. CAT activity was expressed as U g⁻¹ FW min⁻¹. Glutathione reductase (GR) activity was assayed according to Foyer and Halliwell (1976). The reaction buffer (1.0 mL) consisted of 25 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.2 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 100 µL enzyme extract. The reaction was started by the addition of GSSG and enzyme activity was determined using the molar extinction coefficient (6.2 mM⁻¹ cm⁻¹) for NADPH. One unit of GR activity was defined as 1.0 g tissue proteins consumed. 1 µmol NADPH at 340 nm for 1 min. GR activity was expressed as U g⁻¹ protein min⁻¹. The content of H₂O₂ was determined according to the method described by Patterson et al (1984) with minor modifications. The fresh leaf samples of mung bean (500 mg) were homogenized in 5 mL ice cold acetone and centrifuged at 10000 g at 4°C for 10 min. Titanium sulphate (TiS2O8; 5%, w/v) and ammonia were added to supernatant and centrifuged at 10000 g at 4°C for 10 min. The precipitate was washed five times with ice acetone by resuspension, drained, and dissolved in 3 mL of 10% (v/v) H₂SO₄. The reaction was monitored by the absorption of titanium peroxide complex at 410 nm with a UV/Vis spectrophotometer (UV-1800, Shimadzu, Japan) according to a standard curve. The content of H₂O₂ was expressed as µmol g⁻¹ FW. GPX

activity was assayed according to the method described by Hemeda and Klein (1990). A 100 mL of reaction mixture contained 10 mL of 1% guaiacol (v/v), 10 mL of 0.3% H₂O₂ and 80 mL of 50 mM phosphate buffer (pH=6.6). 75 µL of enzyme extract was added to reaction mixture with a final volume of 3 mL. An increase in absorbance due to guaiacol oxidation was monitored at 470 nm for 3 min and enzyme activity was expressed as U g⁻¹ FW min⁻¹.

First Kolmogorov-Smirnov test was used to check for data normality and the Levene test for homogeneity of variance in SPSS for windows 7. All the original datasets was found to be conformed to a normal distribution. When necessary, depend variables were transformed using the natural logarithmic to achieve requirements of homogeneity of variance (P<0.05). Analysis of data was performed using SPSS software and Duncan mean comparison at level of 5% (Produced by SPSS Inc., it was acquired by IBM in 2009, Version 16). From office software collection (2013) Excel program used to draw figures.

RESULTS AND DISCUSSION

Eco - physiological assay

Compounded variance analysis of traits displayed that based on the number of leaves per plant, the main effects of year, drought stress and the effects of inoculation, drought stress × inoculation were significant (P<0.05 and P<0.01 respectively) (Table 2). According to interaction effects of drought × inoculation, the highest and lowest number of leaves in plant were obtained in control plus *G. mosseae* and in cutting irrigation in pod filling stage plus *G. mosseae* treatments with 30.4 and 16.02 leaves respectively (Fig 1a). Drought stress reduced the number of leaves per plant and it was increased by application of *G. mosseae* by influence on physiological and biological activities of plant cells (Table 3). Reduction in plant leaves is associated with decline in cell magnitude and increase aging of leaves under water stress condition. Leaf growth and leaf surface was significantly reduced with water deficiency (Table 3). In order to overcome these effects, plants develop antioxidant defense systems comprising both enzymatic and non-enzymatic components that serve to prevent ROS accumulation and alleviate the oxidative damage occurring during drought stress (Miller et al., 2010).

Chlorophyll content of leaves under stopping irrigation in pod filling stage was 17.60% higher than control plants (Table 3). *G.mosseae* and *P.fluorescens* 169 had high and insignificant effect on increasing the Chlorophyll content of mung bean. Combination of *G.mosseae* and *P.fluorescens* 169 increased chlorophyll content of plants with 1.79% over control plants (Table 3). AMF enhances photosynthetic rate, stomata conductance and water use efficiency of *Leymus chinensis* by 63%, 38% and 15% respectively (Li et al., 2019).

Relative of water content (RWC) has been introduced as a useful indicator of the equilibrium of plant 's water. Measurements of water use efficiency provide a combination of water use rates and then allow more

discussion about plant relationships when water is limited. The results of combined analysis of variance showed that the effect of year on drought stress based on relative water content was significant (P< 0.05) (Table 2). The lowest relative water content obtained from imposed drought stress in flowering stage in 2016 with average 39.9 % (Table 3). Reduction in relative water content of leaves by imposing drought stress reported by Moghadasan et al., (2016) and Aliasghar zad et al., (2006). It seems that decreasing the relative leaf content due to water shortage is related to the closure of stomata by releasing abscisic acid hormone so that under drought stress condition it is produced in root cells and accumulated in stomata cells (Khan et al., 2008).

Combined analysis of variance showed that the differences between the main level of year and bilateral effects of year × replication and year × inoculation and between the effects of inoculations were significant (P<0.05) in term of seed phosphorous (Table 2). The maximum amount of phosphorous was observed in plants inoculated with *G. mosseae* (68.19%) which was higher than controlled plants and other treatments (Table 3). In 2017 inoculation by *G.mosseae* had more efficiency to increase seed phosphorous level as compared with 2016 (Table 3). It seems that the reduction of moisture stored in the soil, the volume and extension of roots in the soil and availability of nutrients in the soil in 2016 was higher than 2017. Therefore, accumulation of phosphorous decreased in 2016. Increasing the amount of phosphorous in seeds inoculated by mycorrhiza species was also reported by Kirda (2002).

Source of variance of traits displayed significant differences between main levels of year, drought stress and interaction effects of year × inoculation (P<0.0 1) (Table 2). Imposed drought stress reduced final seed yield of mung bean. The lowest amount of seed yield obtained in stopping irrigation in pod filling stage with average of 391.4 kg/ha (Table 3). According to effects of year × inoculation, inoculation of plant by *G.mosseae* in 2017 caused the highest seed yield with average of 665 kg/ha. High nutrients availability of plant due to high growth and root colonization was the main mechanism to improve seed yield of mung bean in drought stress condition (Moradi et al., 2011). Co inoculation of *G. mosseae* and *P. fluorescens*169 was more effective to increase seed yield in water deficiency condition in comparison with control treatment. *Pseudomonas* is one of the most important bacteria strain to increase plant growth under drought condition (Nori et al., 2016).

Variance analysis of traits resulted that differences between main levels of drought stress and inoculation based on root colonization was significant (P<0.01) (Table 2). Root colonization percentage of plants in normal irrigation condition was high with average of 56.29 %. (Table 3). Inoculation by both *G. mosseae* and *P. fluorescens*169 caused the highest root colonization in 2017 (Table 3 and Fig 1b). When plant is growing actively, colonization of root by arbuscular mycorrhiza increased. Plants inoculated by *G. mosseae* had the highest root

colonization percentage (by 61.94 %). In drought imposed condition plants inoculated by both *G. mosseae* and *P. fluorescens* 169 was more effective to increase root colonization of mung bean (Table 3). The differences in root colonization of mung bean in 2016 and 2017 was due to

significant reduction in germination and hyphae growth. In all, plants with high root colonization had high efficiency to uptake water, phosphorous and consequently increased seed yield.

Table 2. Significant of mean squares (F values) of eco-physiological traits of mung bean

Sources of variation	Mean squares						
	Degree of freedom	Number of leaves in plant	Chlorophyll content	Relative water content	Phosphorous content of seeds	Seed yield	Root colonization
Year	1	**	ns	ns	**	**	*
Year × replication	4	**	ns	ns	**	ns	ns
Drought	2	**	*	*	ns	**	**
Year × Drought stress	2	ns	ns	*	ns	ns	ns
Inoculation	3	*	*	*	*	ns	**
Year × Inoculation	3	ns	ns	ns	**	**	ns
Drought × Inoculation	6	*	ns	ns	ns	ns	*
Year × Drought × Inoculation	6	ns	ns	ns	ns	ns	ns
Error	44						

ns, *and ** non-significant, significant in probability level of 5% and 1% respectively

Table 3. Mean comparison of eco-physiological traits of mung bean

Traits	Number of leaves per plant	Chlorophyll content	Relative water content (%)	Phosphorous content of seed (%)	Seed yield (kg/ha)	Root colonization (%)
Year						
2016	23.6 ^a	47.2 ^a	50.6 ^a	9.7 ^b	379.2 ^b	37.9 ^b
2017	18.01 ^b	49.8 ^a	49.8 ^a	23.07 ^a	564.7 ^a	58.8 ^a
Drought						
Normal irrigation (control)	25.2 ^a	46.30 ^c	46 ^c	18.32 ^a	577.9 ^a	56.29 ^a
Stopping-irrigation in flowering stage (D ₁)	20.7 ^{ab}	50.2 ^b	50.51 ^b	15.25 ^a	446.5 ^b	45.84 ^b
Stopping-irrigation in pod setting stage (D ₂)	16.4 ^b	54.1 ^a	54.1 ^a	15.61 ^a	391.4 ^c	42.94 ^b
Inoculations						
Non-inoculation	18.87 ^b	45.2 ^c	47.4 ^d	15.27 ^b	481.7 ^a	41.40 ^c
<i>Glomus mosseae</i>	22.28 ^a	50.6 ^a	50.09 ^b	19.68 ^a	485.8 ^a	51.66 ^b
<i>Pseudomonas fluorescens</i> 169	22.30 ^a	52.1 ^a	54.2 ^a	14.49 ^b	453.02 ^b	40.26 ^c
<i>G. mosseae</i> + <i>P. fluorescens</i> 169	19.81 ^b	46.01 ^b	49.1 ^c	16.15 ^b	467.2 ^b	60.11 ^a

In each column the same letter (s) indicates non-significant difference among treatments (Duncan's test, P<0.05)

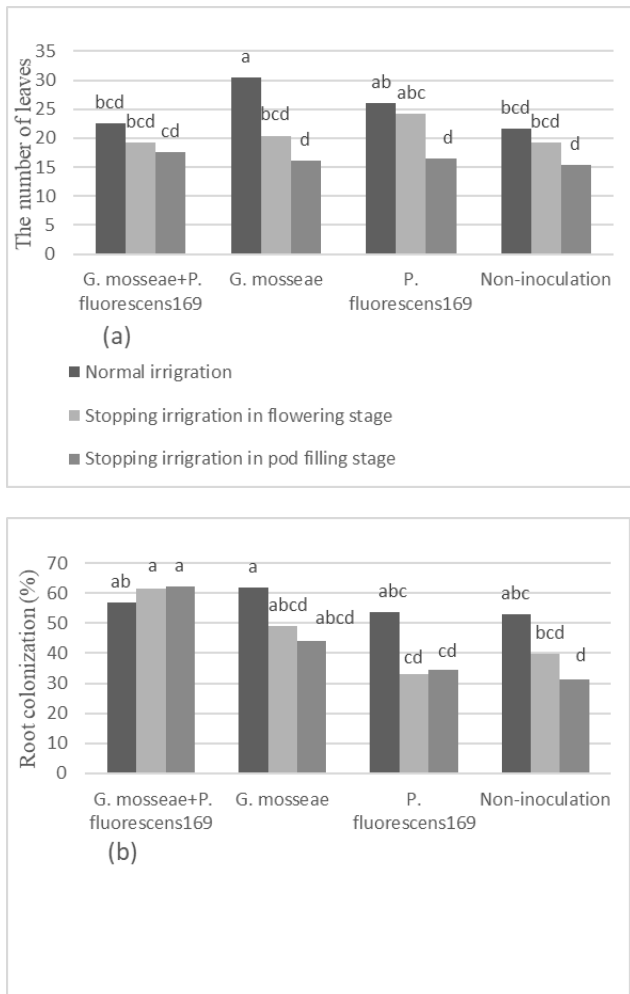


Fig 1. Comparison of the number of leaves (a), root colonization (b) based on interaction effects of drought and inoculation treatments. Means followed by same letter (s) are not significantly different at Duncan's 5%.

Antioxidant assay

According to Table 4, there were high and significant differences between Catalase activities (CAT) of plants based on main effects of year, drought, inoculation and interaction effects of drought \times inoculation ($P < 0.01$). CAT in plants inoculated with both *G. mosseae* plus *P. fluorescens* 169 was higher than single inoculated treatments. Co-inoculation of *G. mosseae* and *P. fluorescens* 169 enhanced catalase activity about 41.28% as compared to the control plants (Table 6). CAT of plant by imposing drought stress increased. But, the highest value was observed in imposing drought stress at flowering stage which was higher than in control plants (Table 5). CAT in all inoculated plants under different water stresses was higher than non-inoculated plants (Fig 2a). Synergistic effect of *G. mosseae* and *P. fluorescens* 169 in stopping irrigation at flowering stage caused 141.3% increase in catalase activity in comparison with control plants (Table 6). CAT is a major enzyme detoxifying hydrogen peroxide under water stress (Heidari and Golpayegani, 2011; Mittler, 2002).

Inoculation had significantly an effect on GR activity of mung bean leaves (Table 4). The effect of *G. mosseae* increasing GR activity of mung bean was 2fold of *P. fluorescens* 169 as compared with non-inoculated plants. However, inoculation with both *G. mosseae* plus *P. fluorescens* 169 caused the highest GR activity of leaves (Table 5). Two year field experiments showed that inoculation with both *G. mosseae* and *P. fluorescens* 169 induced GR activity of leaves and this increase in 2017 was about 9.09% higher than in 2016 (Table 6). GR enzyme activity showed that amount of this enzyme by cutting irrigation in flowering stage and pods filling stage treatment plots were 81.09% and 63.02% respectively as compared with normal irrigated plants (Table 5). Combination of *G. mosseae* and *P. fluorescens* 169 enhanced GR activity in the leaves of plants in cutting irrigation in flowering stage (Fig 2b). Glutathione reductase plays an important role in adaptation to oxidative stress. This enzyme (GR) is responsible for conversion of oxidized glutathione to reduced glutathione and maintain a high ration of GSH to GSSG. Glutathione in Xanthophyll, Mehler and Ascorbate- Glutathione cycles has the main role on hydrogen peroxide collection and preservation of GSH. Hence, increased GR due to restoration of oxidized glutathione is very important (Sairam et al., 2003).

Hydrogen peroxide (H_2O_2) content of leaves significantly decreased in inoculated plants as compared with non-inoculated ones. This reduction in plants inoculated by *P. fluorescens* 169 was the highest while *G. mosseae* and combination of *G. mosseae* plus *P. fluorescens* 169 had the same effect on H_2O_2 decline (Table 5). In addition, the efficiency of *P. fluorescens* 169 in reduction of H_2O_2 was 41.6% and 21.4% higher than control and other inoculated treatments, respectively (Table 5).

H_2O_2 content of leaves was increased by imposing drought stress. Stopping irrigation in pod filling stage caused the highest H_2O_2 content of leaves by 44.96% as compared with control plants (Table 5). According to Fig 2c, non-inoculated plants in all drought stressed treatments had the highest average of hydrogen peroxide content. This result indicates that inoculation with *G. mosseae*, *P. fluorescens* 169 and both could help plants to reduce the oxidative damage caused by drought stress.

Imposing drought stress by stopping irrigation in both flowering and pod filling stages significantly increased GPX activity over the control (Table 5). Inoculation treatments had significant effect on the activity of GPX. Thus, mix inoculation by *G. mosseae* and *P. fluorescens* 169 caused the highest GPX by 134.61% (Table 5). Based on interaction effects of inoculation and drought stress, the synergistic effect of *G. mosseae* and *P. fluorescens* 169 under stopping irrigation in pod filling stage induced the highest GPX enzyme activity (Fig 2d).

Table 4. Mean squares of measured antioxidative enzymes and H₂O₂ of mung bean

S.O.V	df	Mean squares			
		CAT	GR	H ₂ O ₂	GPX
Year	1	**	**	**	**
Year× replication	4	ns	ns	**	ns
Drought	2	**	**	**	**
Year × Drought stress	2	ns	**	**	**
Inoculation	3	**	**	**	**
Year × Inoculation	3	ns	**	*	**
Drought × Inoculation	6	**	**	**	**
Year× Drought × Inoculation	6	ns	*	ns	**
Error	44				
Coefficient of variation (%)		0.98	7.15	5.05	5.40

ns, *and ** non-significant, significant in probability level of 5% and 1% respectively.

Sources of variation (S.O.V), Degree of freedom (df), Catalase (CAT), Glutathione reductase (GR), hydrogen peroxidase (H₂O₂), glutathione peroxidase (GPX)

Table 5. Mean comparison of measured antioxidative enzymes and H₂O₂ based on inoculation and drought

Inoculation	CAT (U g ⁻¹ FWmin ⁻¹)	GR (U g ⁻¹ protein min ⁻¹)	H ₂ O ₂ (μmol)	GPX (U g ⁻¹ FW min ⁻¹)
Non-inoculation	16.81 ^d	0.18 ^d	0.24 ^a	0.26 ^d
<i>Glomus mosseae</i>	22.35 ^b	0.36 ^b	0.17 ^b	0.49 ^b
<i>Pseudomonas fluorescens</i> 169	20.05 ^c	0.27 ^c	0.14 ^c	0.38 ^c
<i>G. mosseae</i> + <i>P. fluorescens</i> 169	23.75 ^a	0.57 ^a	0.17 ^b	0.61 ^a
Drought	CAT	GR	H ₂ O ₂	GPX
Normal irrigation	15.37 ^c	0.238 ^c	0.149 ^c	0.240 ^b
Stopping irrigation in flowering stage (D ₁)	23.75 ^a	0.431 ^a	0.189 ^b	0.529 ^a
Stopping irrigation in pod filling stage (D ₂)	23.10 ^b	0.388 ^b	0.216 ^a	0.544 ^a

In each column the same letter (s) indicates non-significant difference among treatments (Duncan's test, P<0.05).

Catalase (CAT), Glutathione reductase (GR), hydrogen peroxidase (H₂O₂), glutathione peroxidase (GPX).

Table 6. Mean comparison of measured antioxidative enzymes and H₂O₂ based on interaction effect of year × inoculation and year × drought

Year	Inoculation	GR (U g ⁻¹ protein min ⁻¹)	H ₂ O ₂ (μmol)	GPX (U g ⁻¹ FW min ⁻¹)
2016	Non-inoculation	0.18 ^e	0.24 ^a	0.35 ^c
	<i>Glomus mosseae</i>	0.32 ^{bc}	0.20 ^b	0.65 ^b
	<i>Pseudomonas fluorescens</i> 169	0.24 ^{cde}	0.15 ^d	0.51 ^c
	<i>G. mosseae</i> + <i>P. fluorescens</i> 169	0.55 ^a	0.18 ^c	0.78 ^a
2017	Non-inoculation	0.19 ^{de}	0.25 ^a	0.17 ^h
	<i>Glomus mosseae</i>	0.41 ^b	0.14 ^d	0.32 ^f
	<i>Pseudomonas fluorescens</i> 169	0.31 ^{bcd}	0.12 ^e	0.25 ^g
	<i>G. mosseae</i> + <i>P. fluorescens</i> 169	0.60 ^a	0.15 ^d	0.43 ^d
Drought	GR	H ₂ O ₂	GPX	
2016	Normal irrigation	0.199 ^d	0.137 ^d	0.318 ^c
	Stopping irrigation in flowering stage (D ₁)	0.424 ^a	0.211 ^{ab}	0.697 ^a
	Stopping irrigation in pod filling stage (D ₂)	0.351 ^b	0.246 ^a	0.714 ^a
20017	Normal irrigation	0.277 ^c	0.161 ^{cd}	0.161 ^d
	Stopping irrigation in flowering stage (D ₁)	0.437 ^a	0.167 ^{cd}	0.361 ^b
	Stopping irrigation in pod filling stage (D ₂)	0.426 ^a	0.186 ^{bc}	0.374 ^b

In each column the same letter (s) indicates non-significant difference among treatments (Duncan's test, P<0.05).

Glutathione reductase (GR), hydrogen peroxidase (H₂O₂), glutathione peroxidase (GPX).

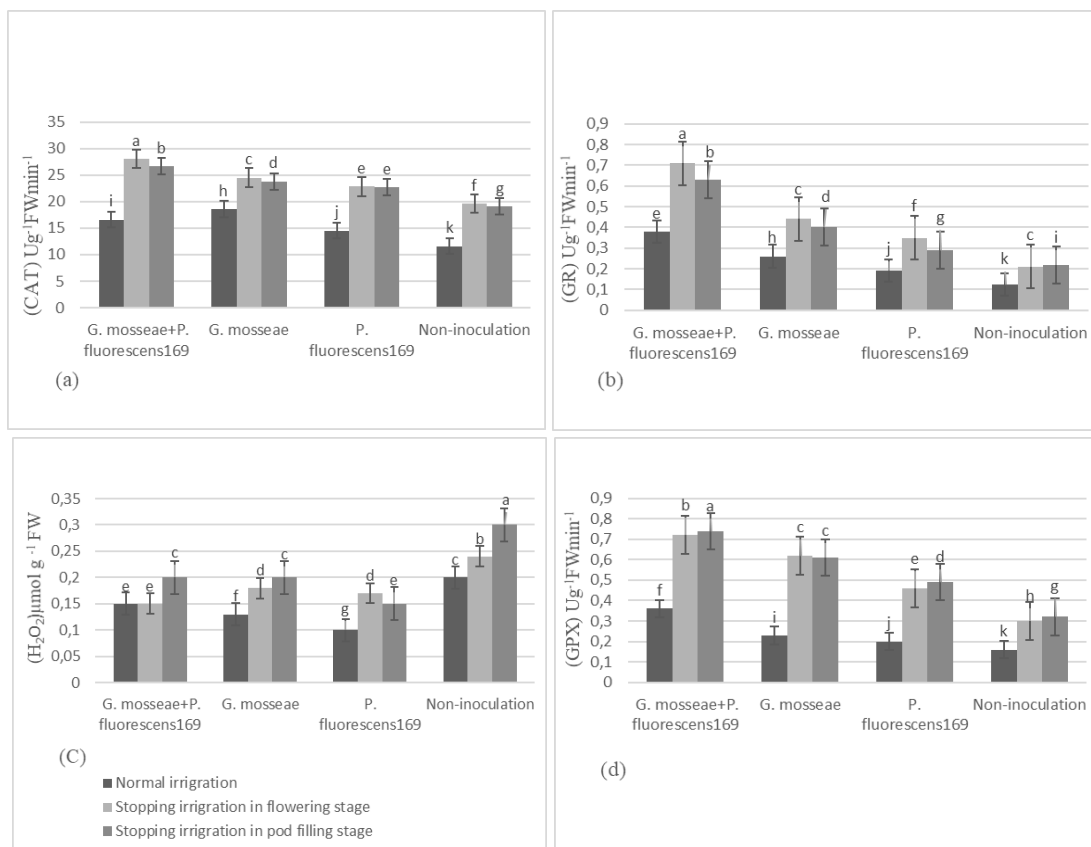


Fig 2. Mean comparison of CAT activity (a) GR activity (b) H₂O₂ content (c) and GPX activity (d) in leaves based on interaction effects of inoculation and drought stress. Means followed by same letter (s) are not significantly different at Duncan's 5%.

CONCLUSION

Pod formation stage of mung bean was distinguished as susceptible growth stage of plant in water deficit condition. In addition, stopping irrigation in pod formation stage had high negative influence on seed yield. The synergistic effects of *G. mosseae* and *P. fluorescens* 169 increased root colonization of mung bean in irrigation condition. According to relative water content, flowering stage in 2016 was sensitive to drought stress, while relative water content of plant in drought condition was higher than normal irrigation condition in 2017. The exact mechanism for mung bean to alleviate harm effects of drought stress was through enhancement of GPX and CAT enzymes by interaction symbiosis between *G.mosseae* and *P.fluorescens* 169 rather than individual ones. It was conclude that relying on APX and GPX is the best strategy improving the tolerance of mung bean under drought stress condition. Stopping irrigation in flowering stage increased CAT, GR and GPX activity of leaves while the highest H₂O₂ content observed in plants under cutting irrigation in pod filling stage. Mixed inoculation of *G.mosseae* and *P.fluorescens* 169 had more beneficial effect on enzyme activities of mung bean under drought stressed condition. In all, the recommendation is using the combination of *G.mosseae* plus *P.fluorescens* 169 as a biological fertilizer in dry area to increase drought tolerance and seed yield of mung bean.

LITERATURE CITED

- Abdul Jaleel, C., P. Manivannan, B. Sankar, A. Kishorekumar, R. Gopi, R. Somasundaram and R. Panneerselvam. 2007. *Pseudomonas fluorescens* enhances biomass yield and ajmalicine production in *Catharanthus roseus* under water deficit stress. *Colloids and Surfaces B: Biointerfaces*. 60 (1): 7-11. <https://doi.org/10.1016/j.colsurfb.2007.05.012>.
- Aliasgharzad, N., M.R. Neyshabouri and G. Salimi. 2006. Effects of arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* on drought stress of soybean, *Biologia*. 67: 324-328.
- Balestrini, R. and E. Lumini. 2018. Focus on mycorrhizal symbioses. *Appl. Soil Ecol.* 123. PP: 299-304. [https://DOI: 10.1016/j.apsoil.2017.09.001](https://DOI:10.1016/j.apsoil.2017.09.001).
- Barea, J.M., R. Azcon and C. Azcon-Aguilar. 2002. Mycorrhizosphere interactions to improve plant fitness and soil quality. *AntonyVan Leeuwenhoek*. 81: 343-351.
- Bashan, Y., A.K. Kamnev and L.E. de-Bashan. 2013. Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. *Biol Fertil Soils*. 49: 465-479.
- Benton, J.J. 2001. Laboratory guild for conducting soil test and plant analysis. 363 P.P. USA. CRC Press. P: 384.
- Biancetto, V., D. Minerdi, S. Perotto and D. Bonfante. 1996. Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria. *Protoplasma*. 193:123-137.
- Dai, A. 2011. Drought under global warming: a review. *Wires Clim. Chang.* 2. pp: 45-5. DOI: 10.1002/wcc.81.
- Farooq, M., M. Hussain, H. Kadambot and M. Siddique. 2014. Drought stress in wheat during flowering and grain filling

- periods. *Critical reviews in plant sciences*. 33(4): 331-349. <https://doi.org/10.1080/07352689.2014.875291>.
- Foyer, C.H. and B. Halliwell. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*. 133 (1): 21–25. doi: 10.1007/BF00386001 PMID:24425174
- Gamalero, E., A. Trotta, N. Massa, A. Copetta, A.G. Martinotti and G. Berta. 2004. Impact of two *fluorescent pseudomonas* and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. *Mycorrhiza*.14:185-192.
- Ganbari, M. and Sh. Mollashahi Javan. 2015. Study the response of mung bean genotypes to drought stress by multivariate analysis. *International Journal of Agriculture Innovations and Research*. 3(4): 2319-1473.
- Giovannetti, M. and B. Mosse. 1980. An evaluation of technique to measure vesicular arbuscular mycorrhizal infection in roots. *New Phytologist*. 84: 489-500.
- Heidari, M. and A. Golpayegani. 2011. Effects of water stress and inoculation with plant growth promoting rhizobacteria (PGPR) on antioxidant status and photosynthetic pigments in basil (*Ocimum basilicum* L.). *Journal of the Saudi Society of Agricultural Sciences*. (11): 57-61.
- Hemeda, H.M. and B.P. Klein. 1990. Effects of naturally occurring antioxidants on peroxidase activity of vegetable extracts. *J Food Sci*. 55(1):184–185.
- Khan, M.S., A. Ziadi, P.A. Wani and M. Oves. 2008. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environmental chemistry letters*: 7: 1-19.
- Kim, Y-C., B.R. Glick, Y. Bashan and C.M. Ryu. 2012. Enhancement of plant drought tolerance by microbes. In: Aroca R (ed) *Plant responses to drought stress: from morphological to molecular features*. Springer Verlag, Berlin & Heidelberg, Germany pp. 383–413.
- Kirda, C. 2002. Deficit irrigation scheduling based on plant growth stages showing water stress tolerance. PP: 3-10. In FAO, water report No.22, *Deficit Irrigation practices*.
- Lau, J.A. and J.T. Lennon. 2011. Evolutionary ecology of plant–microbe interactions: soil microbial structure alters selection on plant traits. *New Phytol*. 192: 215–224.
- Lenoir, I., J. Fontaine and L.H. Sahaoui. 2016. Arbuscular mycorrhizal fungal responses to abiotic stresses: a review. *Phytochemistry*. 123: 4-15. <https://doi.org/10.1016/j.phytochem.2016.01.002>.
- Li, J., B. Meng, H. Chai, X. Yang, W. Song, S. Li, A. Lu, T. Zhang and W. Sun. 2019. Arbuscular Mycorrhizal fungi alleviate drought Stress in C₃ (*Leymus chinensis*) and C₄ (*Hemarthria altissima*) grasses via altering antioxidant enzyme activities and photosynthesis. *Front. Plant Sci*. 10: 499. doi: 10.3389/fpls.2019.00499.
- Lies, L., A. Delteil, Y. Prin and R. Duponnois. 2018. Using Mycorrhiza helper microorganisms (MHM) to improve the mycorrhizal efficiency on plant growth. In: Meena V. (eds) *Role of Rhizospheric microbes in soil*. Springer, Singapore. pp: 277-298. https://doi.org/10.1007/978-981-10-8402-7_11
- Miller, G., N. Susuki, S. Ciftci-Yilmaz and R. Mittler. 2010. Reactive oxygen species homeostasis and signaling during drought and salinity stresses. *Plant Cell Environ*. 33: 453–467.
- Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*. 7: 405–410.
- Moghadasan, Sh., A. Safipour Afshar and F.S. Nematpour. 2016. The role of Mycorrhiza drought tolerance of marigold (*Calendula officinalis* L.). *Journal of Crop Eco physiology*. 9(4): 521-532.
- Mohamed, O., A.E. Fouad, B. Laila and Q. Ahmed. 2014. Effectiveness of arbuscular mycorrhizal fungi in the protection of olive plants against oxidative stress induced by drought. *Spanish Journal of Agricultural Research*. 12(3): 763-771.
- Moradi, M., A. Siadat, K. Khavazi, R. Naseri, A. Maleki and A. Mirzaei. 2011. Effect of application of bio-Fertilizer and phosphorous fertilizer on quantities and qualitative traits of spring wheat. *Journal of Crop Eco physiology*. 5(18): 51-66.
- Nori, M.J., A. Mozafari and M. Mirzaee- Haydari. 2016. Evaluation of yield and yield components of lentil (*Lens Culuaris* Medik) effected by biofertilizer, nitrogen starter and supplemental irrigation on Kermanshah province, *Journal of Agronomy and plant Breeding*. 12(1): 1-14.
- Notor, G. and C.H. Foyer. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 49: 249–279.
- Patterson, B.D., E.A. MacRae and I.B. Ferguson. 1984. Estimation of hydrogen peroxide in plant extracts using titanium (IV). *Anal Biochem*. 139(2):487–492. PMID: 6476384
- Rahimzadeh, S. and A.R. Pirzad. 2019. *Pseudomonas* and *mycorrhizal* fungi co-inoculation alter seed quality of flax under various water supply conditions. *Industrial crops and products*. 129: 518-524. <https://doi.org/10.1016/j.indcrop.2018.12.038>
- Rasool, S., A. Ahmad, T.O. Siddiqi and P. Ahmad. 2013. Changes in growth, lipid peroxidation and some key antioxidant enzymes in chickpea genotypes under salt stress. *Acta Physiol Plant*. 35: 1039-1050.
- Roland, F., J.R. Beers and W.S. Irwin. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem*. 195: 133-140.
- Ruiz-Lozano, J.M. and P. Bonfante. 2001. Intracellular Burkholderia strain has no negative effect on the symbiotic efficiency of the arbuscular mycorrhizal fungus *Gigaspora margarita*. *Plant Growth Regulation*. 34: 347–352.
- Sairam, R.K., K.V. Rao and G.C. Srivastava. 2003. Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. *Plant Science* 163:1037-1046.
- Sardans, J. and J. Peñuelas. 2004. Increasing drought decreases phosphorus availability in an evergreen Mediterranean forest. *Plant Soil*. (267): 367-377. <https://doi.org/10.1007/s11104-005-0172-8>.
- Steven, W., T. Ritchie, H. Nguyen and A.S. Holaday. 1990. Leaf water content and gas exchange parameters of two wheat genotypes differing in drought resistance. *Crop Sci*. 30(1):105-177.
- Toker, C., and N. Mutlu. 2011. *Biology and breeding of food legumes*, ed. Pratap, A and J. Kumar. PP: 241-261. CABI.
- Toker, C., J. Gorham and M.I. Cagirgan. 2009. Certain ion accumulations in barley mutants exposed to drought and salinity. *Turkish Journal of Field Crops* 14 (2): 162-169.
- Zhang, J.X. and M.B. Kirkham. 1994. Drought stress induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. *Plant Cell Physiol*. 35: 785–791.
- Zhang, Z.F., J. Zhang, G. Xu, L. Zhou, and Y. Li. 2019. Arbuscular mycorrhizal fungi improve the growth and drought tolerance of *Zenia insignis* seedlings under drought stress. *New Forests*. 50(4): 50- 593. <https://doi.org/10.1007/s11056-018-9681-1>